Disposition of Acetaminophen and Indocyanine Green in Cystic Fibrosis-Knockout Mice

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ABSTRACT Drug treatment poses a therapeutic challenge in cystic fibrosis (CF) because the disposition of a number of drugs is altered in CF. Enhanced clearance of acetaminophen (APAP) and indocyanine green (ICG) have previously been reported in CF patients. The objective of the current study was to investigate if the CF-knockout mouse model ($cftr^{m1UNC}$) shows altered pharmacokinetics similar to those seen in CF patients using the 2 model compounds APAP and ICG. Clearance (CL/F) of APAP and renal (CL_R) and formation (CL_f) clearance of acetaminophen glucuronide (AG) and acetaminophen sulfate (AS) were determined in CF-knockout mice following administration of APAP (50 mg/kg, intraperitoneal). CL_R of AS was 19.5 and 12.9 (mL/min per kg) and CL_f of AS was 10.4 and 6.7 mL/min per kg for homozygous and heterozygous males, respectively, which was significantly different between groups. CL_R of AG was 6.3 and 4.8 mL/min per kg and CL_f of AG was 9.6 and 8.9 mL/min per kg for homozygous and heterozygous males, respectively, although not reaching statistical significance. No significant differences were noted in either Cl_R or CL_f of AG and AS in female CF mice. Plasma concentrations of ICG (10 mg/kg, intravenous) were determined over 0 to 15 minutes. Homozygous females showed a higher apparent volume of distribution (96 mL/kg) relative to heterozygous females (72 mL/kg). Similar to CF patients, a trend toward a lower C_{max} was noted in homozygous male and female mice. However, contrary to human data, no significant differences in CL of ICG were noted. These results suggest that the CF-knockout mice have potential as a model for studying altered drug disposition in CF patients.

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INTRODUCTION

Cystic fibrosis (CF) is the most common fatal genetic disease in the Western world. In the United States, approximately 30,000 people are living with the disease, and there are 1,000 new cases diagnosed each year. About 5% of Caucasians are asymptomatic carriers, and 1 child in approximately 2,500 of European descent carries 2 defective copies of the gene and has the disease (1). In general, altered pharmacokinetics of numerous drugs in the CF population include increased volume of distribution (Vd), decreased plasma concentrations, and enhanced renal and non-renal clearance of drugs.

The mechanistic basis for the altered pharmacokinetics in CF is unknown, although alterations in hepatic metabolism, blood flow, or transport have been suggested (2-4). Determining a mechanism for altered pharmacokinetics of drugs in CF is unlikely if experimentation is limited to clinical studies. The use of an appropriate animal model to study this phenomenon is highly desirable because it would permit more invasive investigations and provide the ability to perform biochemical studies with isolated tissues.

In the present study, we test the hypothesis that the CF-knockout mouse can be used as an animal model

to predict the altered pharmacokinetics of drugs in CF. Increased clearance of acetaminophen (APAP) and indocyanine green (ICG) has been reported in CF patients (2-5). In order to test the above hypothesis, pharmacokinetics of 2 model compounds, APAP and ICG, were studied in CF-mice. Results from the study indicate that CF-knockout mice appear promising for studying the altered pharmacokinetics in CF.

MATERIALS AND METHODS

Animals

$Cftr^{m1UNC}$ -knockout mice generated at the University of North Carolina-Chapel Hill animal facility were used in this study and are a hybrid strain containing genetic material from C57BL/6, 129/SvEv, Balb/c, and DBA/2 mice (6). $Cftr^{m1UNC}$-knockout mice breed and pass on the defective *cftr* gene in a simple Mendelian pattern (7). $Cftr^{m1UNC}$-knockout mice are maintained on Colyte (PEG 3350 and electrolytes) to prevent intestinal obstructions, resulting in soft stools without diarrhea (6).

Genotyping the $cftr^{miUNC}$-knockout mice

Tail DNA from the CF-mice was isolated and stored at 2 to 8°C in Tris-EDTA using the protocol of Miller et al, 1988 (8). Mice were then genotyped by polymerase chain reaction (PCR) (Robocycler; Stratagene, La Jolla, CA) using a protocol provided by the Cystic Fibrosis Center, University of North Carolina-Chapel Hill. Primers used were 67 Common primer (CAG TGA AGC TGA GAC TGT GAG CTT), 3215+ (CTG TAG TTG GCA AGC TTT GAC), and 45- (ACA CTG CTC GAG GGC TAG CCT CTT C). Products of the PCR were run on a 1% agarose gel, followed by staining with ethidium bromide, and were viewed on an ultraviolet transilluminator.

Materials

APAP, acetaminophen glucuronide (AG), 3-acetamidophenol, and ICG were purchased from Sigma Chemical (St. Louis, MO). Acetaminophen sulfate (AS) was generously provided by Dr. Marilyn Morris (State University of New York at Buffalo, Buffalo, NY).

Animal Treatment

For studies with APAP, adult mice ($cftr^{m1UNC}$) were housed individually in metabolism cages with free access to food and water. Each mouse was administered 50 mg/kg of APAP as a 10 mg/mL solution. Urine was collected over 18 hours, then centrifuged to remove solid contaminants. The volume of the supernatant was measured then frozen at -20°C. After a washout period of 7 days, the same mice were injected with APAP (50 mg/kg, intraperitoneal [ip]), and blood samples were collected sequentially by tail artery bleeding at 0, 15, 30, 45, 60, 120, and 180 minutes in males and 0, 5, 10, 15, 20, 25, 30, 45, 60, 120, and 180 minutes in females. The samples were then centrifuged, and plasma was frozen at -20°C until high-performance liquid chromatography (HPLC) analysis. For studies with ICG, mice were injected with ICG (10 mg/kg, intravenous [iv]) via the tail vein as a 2 mg/mL solution. The solution was made daily and protected from light to minimize degradation. Blood was collected at 2, 4, 6, 8, 10, and 15 minutes, centrifuged, and the plasma frozen at -20°C prior to assay.

Drug and Metabolite Analysis

The procedure used for the assay of APAP and its 2 major metabolites was similar to that described previously (9). Briefly, a reversed-phase HPLC assay was used for the detection of APAP and its metabolites. The mobile phase was 7% acetonitrile 50 mM sodium sulfate and 50 mM potasssium phosphate buffer with a flow rate of 1.3 mL/min. APAP, AG, and AS were detected at 254 nm. Retention times were approximately 4.5, 6, 8, and 12 minutes, respectively, for AG, AS, and APAP and the internal standard (3-acetamidophenol). For HPLC assay of ICG, the procedure used was similar to that described previously (10,11). Briefly, chromatography was performed on a reversed-phase column (Bondapack C18; Water's Associates, Milford, MA) employing a mobile phase of 50 mM phosphate buffer (50 mM KH_2PO_4:K_2HPO_4; pH = 5.52):acetonitrile (55:45), with a flow of 2 mL/min. ICG was detected at 720 nm and had a retention time of approximately 5.5 minutes.

Free fraction of APAP and its metabolites AG and AS were determined by ultrafiltration (Amicon, Bedford, MA). Plasma from CF mice was spiked with APAP, AG, or AS and transferred to the sample reservoir of the filtration device and centrifuged at 900 *g* for 10 minutes at ambient temperature. Total and free concentrations of APAP, AG, and AS were determined by HPLC.

Pharmacokinetic and Statistical Analysis

Pharmacokinetic analysis was performed using noncompartmental analysis with WinNonlin (Pharsight, Mountain View, CA). Assuming F = 1 for ip APAP, clearance (CL/F) of APAP, renal clearance (CL_R), and formation clearance (CL_f) of acetaminophen glucuronide (AG) and acetaminophen sulfate (AS) were determined. Formation clearances of AS and AG were calculated based on assumptions similar to those employed by the human study; that is, no sequential or renal metabolism occurred, and all metabolite formed was recovered in the urine. Because the disposition profile of ICG was best described by a 1-compartment model, pharmacokinetic parameters (CL, C_{max}, k, and V) were determined by this approach. Pharmacokinetic data are expressed as mean ± SD. Comparison between values was made using a general linear models (glm) followed by least square means analysis. Data were analyzed using Statistical Analysis System (SAS Institute, Cary, NC). The acceptable level of statistical significance was $P \leq .05$.

RESULTS

APAP Pharmacokinetics in the CF Mouse

Pharmacokinetic parameters of APAP (50 mg/kg, ip) determined in male and female CF mice are shown in Tables 1 and 2. A representative plasma profile of APAP and its metabolites AG and AS in a male CF mouse is represented in Fig. 1. Similar to CF patients, significant differences in renal clearance ($CL_{R,AS}$) and clearance of formation ($CL_{f,AS}$) of AS were noted between heterozygous and homozygous CF male mice. However, no significant differences in either total systemic clearance (CL/F, assuming F = 1), renal clearance ($CL_{R,AG}$), or formation clearance ($CL_{f,AG}$) were noted between the homozygous and heterozygous CF mice (Tables 1 and 2). Homozygous male mice did show a trend towards increased renal clearance ($CL_{R,AG}$) and an increased clearance of formation ($CL_{f,AG}$) of AG compared to the heterozygous males (controls) (Table 1), although statistical significance was not obtained in this case. Although a trend toward increased clearance was seen in homozygous females, these females did not differ significantly from the heterozygous females in either CL_R or CL_f for AG and AS (Table 2). Significant differences were noted between male and female CF mice in total clearance (CL/F) and renal clearance of AS ($CL_{R,AS}$) (Tables 1 and 2), which was not unexpected in an inbred strain of mice. Sex-selective expression of different enzymes has already been reported in mice (12,13). Sex differences in APAP sulfation and glucuronidation have been reported in Sprague Dawley rat hepatocytes where increased sulfation of APAP is reported in male rats (14). Such sex-specific differences have been reported for other substrates besides APAP like HMBA (7-hydroxymethyl-12-methyl-benz[a] anthracene). Adult female rat livers showed a much higher cytosolic sulfotransferase activity for HMBA metabolism compared to male rats (15). Sex differences have also been reported in glucuronide metabolism of pirmenol (an anti-arrhythmic drug developed by Warner Lambert/Parke Davis) (16). Other enzymes besides Phase II metabolic enzymes also show such sex-specific differences (12,17). Differences in transporter expression have also been recently reported (18,19). Although not much information regarding sex-specific expression of phenolsulfotransferase (PST), UDP-glucuronyl transferases (UGT), or transporter expression in humans or the $cftr^{m1UNC}$-knockout mice is currently available in literature, it is likely that the sex-specific differences noted for APAP are attributable to differences in expression of specific enzymes involved in metabolism or transport of APAP metabolites. Urinary recovery of APAP was similar to that reported in humans where about 65% to 75% of APAP is excreted as the metabolites AG and AS, (Tables 1 and 2) (5). Free fraction (f_u) of APAP and its metabolites AG and AS in plasma were not measurably different in the small number of samples analyzed (Tables 1 and 2).

Table 1. Pharmacokinetic Parameters for APAP and Its Conjugated Metabolites in Male CF Mice After 50 mg/kg Intraperatoneal Administration*

Parameter	CF Male Mice (-/-)	CF Male Mice (+/-)
CL/F(ml/min/kg)	34.8 ± 2.9[a c]	35.4 ± 4.8[c]
$f_{e, AG}$ (%)	28.5 ± 6.4[c]	25.0 ± 6.4[c]
$CL_{R,AG}$ (ml/min/kg)	6.3 ± 1.3	4.8 ± 3.0
$CL_{f,AG}$ (ml/min/kg)	9.6 ± 2.1	8.9 ± 2.8
$f_{e, AS}$ (%)	19.1 ± 3.3	12.3 ± 6.
$CL_{R,AS}$ (ml/min/kg)	19.5 ± 3.8[b c]	12.9 ± 7.6
$CL_{f,AS}$ (ml/min/kg)	10.4 ± 1.8[b]	6.7 ± 3.4
$f_{u,APAP}$	0.86	0.83
$f_{u,AG}$	0.87	0.82
$f_{u,AS}$	0.68	0.70

**CL/F indicates relative plasma clearance of APAP; fe,AG, fraction of dose recovered in the urine as AG (units are expressed as APAP equivalents); CLR,AG, renal clearance of AG; CLf,AG, formation clearance of APAP to AG; fe,AS, fraction of dose recovered in the urine as AS (units are expressed as APAP equivalents); CLR,AS, renal clearance of AS; CLf, AS, formation clearance of APAP to AS; fu, APAP, free fraction of APAP; fu,AG, free fraction of AG; fu,AS, free fraction of AS.*

aMean ± SD, n = 6.

bSignificant difference between homozygous and heterozygous CF males.

cSignificant difference between CF males and CF females.

Table 2. Pharmacokinetics of APAP and its Conjugated Metabolites in Female CF Mice After 50 mg/kg Intraperitoneal Administration

Parameter	CF Female Mice (-/-)	CF Female Mice (+/-)
CL/F (ml/min/kg)	25.8 ± 2.8[b]	24.8 ± 3.0[b]
$f_{e, AG}$ (mg/kg)	38.1 ± 7.7[b]	33.5 ± 7.9[b]
$CL_{R,AG}$ (ml/min/kg)	4.7 ± 0.8	4.1 ± 2.5
$CL_{f,AG}$ (ml/min/kg)	9.4 ± 1.5	8.2 ± 1.6
$f_{e, AS}$ (mg/kg)	35.7 ± 4.7	30.9 ± 7.2
$CL_{R,AS}$ (ml/min/kg)	8.4 ± 1.4[b]	7.9 ± 2.0
$CL_{f,AS}$ (ml/min/kg)	8.9 ± 1.4	7.5 ± 1.0
$f_{u,APAP}$	0.84	0.87
$f_{u,AG}$	0.85	0.85
$f_{u,AS}$	0.67	0.65

aMean ± SD, n = 6.

bSignificant difference between CF males and CF females.

See Table 1 for parameter definitions.

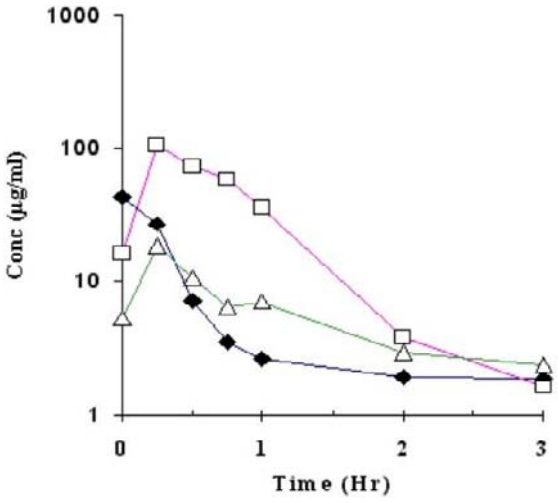

Figure 1. Representative plasma concentration time profile of acetaminophen (APAP) (filled diamond) and its glucuronide (open square) and sulfate (open triangle) metabolites in a cystic fibrosis (-/-) male mouse administered APAP (50 mg/kg, ip).

ICG Pharmacokinetics in the CF Mouse

Following administration of 10 mg/kg ICG, pharmacokinetic parameters were determined (Table 3) with a representative plasma profile (Fig. 2). The disposition profile for ICG was best described by a 1-compartment model. This is similar to the human situation in which a 1-compartmental fit for ICG has been reported in CF patients (3,4). CF-knockout mice did not show any difference in total systemic clearance (CL), unlike the situation in CF patients, wherein increased clearance was reported to correlate with the severity of CF (2). Similar to the results with APAP, gender differences in clearance of ICG were noted in CF mice. Homozygous female CF-mice showed an increased V when compared with the heterozygous mice used as controls (Table 3). These results are in agreement with previous reports of an increase in V for ICG in CF patients (3,4). Similar to CF patients, homozygous CF-mice also showed a trend towards lower peak concentrations of ICG relative to heterozygous mice (Table 3). Blood-to-plasma ratios for ICG were 0.58 ± 0.18 in (-/-) female mice vs. 0.55 ± 0.025 in (+/-) female mice.

Table 3. Pharmacokinetic Parameters for ICG in Male and Female CF Mice After a 10-mg/kg Intravenous Bolus*

Parameter	Male (-/-)	Male (+/-)	Female (-/-)	Female (+/-)
CL/F (ml/min/kg)	23.0 ± 6.0	25.0 ± 7.0	28.0 ± 7.1	30.0 ± 8.5
C_{max} (μ g/ml)	218 ± 71[c]	223 ± 51[c]	112 ± 38[c]	140 ± 14[c]
k (min^{-1})	1.5 ± 0.4[c]	1.4 ± 0.4	2.5 ± 1.0[c]	1.8 ± 0.4
V (ml/kg)	49.0 ± 14[c]	47.0 ± 12[c]	96.0 ± 2.6[b c]	72.0 ± 6.9[c]

**CL indicates total plasma clearance of indocyanine green; V, apparent volume of distribution; K, elimination rate constant.*

aMean ± SD; n = 6.

bSignificant difference between homozygous and heterozygous CF-females.

cSignificant difference between CF males and CF females.

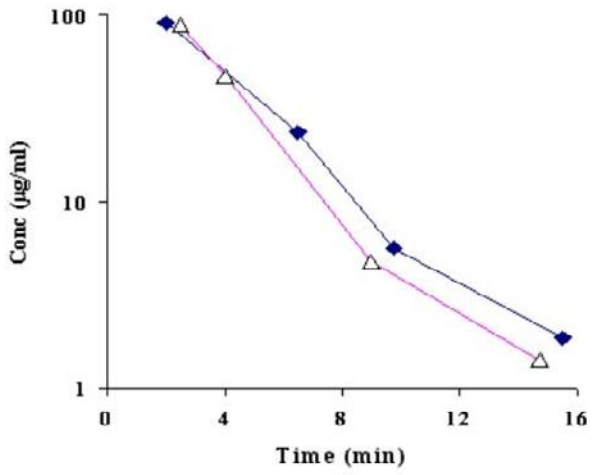

Figure 2. Representative plasma concentration time profiles of indocyanine green (ICG) in cystic fibrosis +/- (open triangle) and -/- (filled diamond) male mice injected with ICG (10 mg/kg, iv) via the tail vein.

DISCUSSION

Studies in CF patients have revealed altered pharmacokinetics for diverse drugs such as gentamicin, tobramycin, dicloxacillin, cloxacillin, theophylline, cyclosporin, APAP, lorazepam, and ICG (3). Alterations in pharmacokinetics include lower plasma concentrations, increased total plasma clearance, and an increase in apparent steady-state volume of distribution. Mechanisms that account for and adequately describe these alterations in CF have not been determined.

Elucidating a mechanism for altered absorption and clearance of drugs is unlikely if experimentation is limited to CF patients. A predictive animal model to study altered drug disposition in CF is therefore desirable. The objective of this study was to evaluate the $cftr^{m1UNC}$ mouse (7) as a potential animal model to predict alterations in pharmacokinetics observed in CF patients, so that compounds that have altered disposition may be identified, and thus drug therapy may be optimized more rationally. This mouse model has altered gastrointestinal and hepatobiliary abnormalities similar to that seen in CF patients (20) but has not previously been evaluated as a model for drug disposition in CF.

Preliminary studies were conducted using 2 model compounds, APAP and ICG, in CF-knockout mice using heterozygous (+/-) littermates as controls. Increased clearance of both APAP and ICG has been reported in CF patients (2,5). Increased clearance of APAP in CF patients has been attributed to greater metabolic clearance of APAP to AG and AS (5). CL/F was found to be 1.5-fold different between CF patients and controls (0.36 vs. 0.25 L/min per kg, respectively) with a 1.7-fold higher CL_f of the glucuronide and sulfate. A trend toward a correlation between the NIH score (index of severity of the disease) and $CL_{f, AG}$ and $CL_{f, AS}$ was also found in CF patients (5).

These preliminary experiments with APAP in CF mice revealed results similar to those seen in humans; ie, a trend of increased $CL_{f,AG}$ and $CL_{f,AS}$, although for the sample size employed, only $CL_{f,AS}$ was significantly different (Tables 1 and 2). Conversion of APAP to its major metabolites via the liver involves 3 distinct steps, uptake, metabolism, and efflux, and alterations in any of these could be responsible for the observed increased clearance.

APAP is a moderately water- and lipid-soluble weak organic acid with a pKa of 9.5, and is largely uncharged at physiological pH. It is reasonable to assume that APAP should be able to cross the cell membrane by simple diffusion alone. However, the presence of a carrier-mediated system that may contribute to uptake at concentrations encountered in vivo has been reported (21). Two metabolic inhibitors, 2,4-dinitrophenol and iodoacetate, reduced the uptake of APAP into hepatocytes, suggesting that uptake of acetaminophen is a combination of a saturable active process and simple diffusion (22).

Conjugation of the drug within the cell is the second step. Because the glucuronidation of APAP at the dose used in the study is unlikely to be rate-limited by the availability of the cofactor UDP glucuronic acid (23,24), increased glucuronidation of APAP in the CF-knockout mice may be attributable to an induction or activation of UGT. Preliminary in vitro studies of APAP glucuronidation using mouse liver microsomes suggest no apparent difference in either Vm or Km for metabolism of APAP by UGT. Increased formation of AS could either arise from an increased hepatic PST activity or from an increase in inorganic sulfate concentration, a precursor to 3-phospho adenosine 5'-phosphosulfate (PAPS), and these options are currently being investigated. However, no differences in inorganic sulfate levels were noted in CF patients, despite a higher $CL_{f,AS}$ (14); therefore, increased inorganic sulfate levels are unlikely to be the cause of higher $CL_{f,AS}$ in the mice.

Transport of the conjugates out of the cell may also be rate-limiting, which would result in an increase in levels of intracellular conjugate and possibly result in product inhibition (25-27). Analogous to rats and humans, CF mice probably excrete AG from hepatocytes via either oatp (organic anion transporting polypeptide), or mrp1 (multidrug resistance associated protein-1) or some as yet unidentified transporter involved in the transport of anions. As with AG, AS is very polar and is a strong acid; therefore, it may also depend on membrane transporters to be excreted from hepatocytes. Previous studies in fetal sheep indicate that AG and AS are not passively transported across the placenta (28); therefore, it is likely that their removal from hepatocytes is facilitated.

Increased clearance of drugs like APAP in CF could arise from an increased expression of transporters, resulting in either increased uptake or efflux of the drug. There is precedence for this statement in that coordinate regulation between expression of cftr and mdr1 has been reported to occur across several species (29-31). A recent study by Trezise et al, 1997 (29) suggests that expression of transporters like mdr1 (multidrug resistance) may be coordinately regulated with ctfr expression. An inverse relationship between cftr and mdr1 expression in the CF mouse with heterozygous mice showing an intermediate level of expression and homozygous CF competent mice (+/+) having lowest levels of mdr has been demonstrated.

ICG is a high extraction ratio probe in humans, which is exclusively cleared by the liver. ICG is efficiently excreted in bile without the need for metabolism, because of its large molecular weight and inherent charge. However, it appears that in the CF-knockout mice, ICG is only a moderate extraction ratio drug (E = 0.6). This conclusion is based on a published value for hepatic blood flow in the mouse of 86 mL/min per kg (32). Because both hepatic blood flow and bile acid uptake have been found to be normal in patients with CF (33), ICG should provide a more direct measure of whether specific hepatic transporters are altered in CF. It is possible that the genetic defect in CF that alters transmembrane regulation of ion flux (ie, cystic fibrosis transmembrane regulator [cftr] protein) may also increase either the cellular uptake of ICG or its eventual secretion into the bile. Following administration of ICG, homozygous CF-knockout mice had results qualitatively similar to CF patients, with a trend toward a decreased C_{max} and an

increased V relative to heterozygous mice. However, in contrast to humans, no difference in CL of ICG was observed in the cftr mice relative to heterozygous controls (Table 3). It is probable that the rate-limiting step in the elimination of ICG, either uptake or efflux, may be different between species.

Increased volume of distribution has been reported for a large number of compounds in CF (eg, antipyrine, lorazepam, and ICG) (2). It has been suggested that in CF patients, chronic reduction in systemic arterial oxygen saturation, which is observed with increasing severity of pulmonary disease, is often associated with increases in both erythrocyte count and plasma volume. These alterations result in an increase in body water/body mass ratio, effectively increasing the available distribution space for drugs that partition to both intravascular and extravascular spaces (2). It should be noted that this difference in V in female CF mice is not attributable to altered blood-to-plasma ratio of ICG. Blood-to-plasma ratios for ICG were not different between homozygous and heterozygous female mice.

In summary, this study shows that, similar to CF patients, CL_f and CL_R of AS is increased in homozygous CF male mice relative to heterozygous (+/-) controls. A trend toward an increased CL_f and CL_R of AG was noted, with no significant differences. This trend may be attributed to the fact that a diverse group of mice (ages 20 to 48 weeks) were used in this study because of limited availability of these mice. Also, (+/+) mice were not used as controls in these studies. The studies by Trezise et al in 1997 (29) indicate that if the increased clearance comes from expression levels of a transporter, then (+/+) mice would serve as better controls. Results with ICG indicate that homozygous CF mice demonstrate a trend toward a decreased C_{max} and an increased V relative to heterozygous mice. However, in contrast to results from CF patients, no difference in CL of ICG is noted in CF mice. The use of (+/+) mice as controls in future studies may increase the ability to discriminate the effects of altered cftr on drug disposition.

We conclude that the CF-knockout mouse model has potential for being employed as an animal model for predicting altered clearance in CF patients. Future studies using (+/+) mice as controls and a wider range of drugs are ongoing.

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